

Apparent Involvement of a Multidrug Transporter in the Fluoroquinolone Resistance of *Streptococcus pneumoniae*

NATALYA N. BARANOVA AND ALEXANDER A. NEYFAKH*

Department of Medicinal Chemistry and Pharmacognosy and Center for Pharmaceutical Biotechnology,
University of Illinois, Chicago, Illinois 60607

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A *Streptococcus pneumoniae* strain selected for resistance to ethidium bromide demonstrated enhanced energy-dependent efflux of this toxic dye. Both the ethidium resistance and the ethidium efflux could be inhibited by the plant alkaloid reserpine. The ethidium-selected cells demonstrated cross-resistance to the fluoroquinolones norfloxacin and ciprofloxacin; this resistance could also be completely reversed by reserpine. Furthermore, reserpine potentiated the susceptibility of wild-type *S. pneumoniae* to fluoroquinolones and ethidium. The most plausible explanation for these results is that *S. pneumoniae*, like some other gram-positive bacteria, expresses a reserpine-sensitive multidrug transporter, which may play an important role in both intrinsic and acquired resistances of this pathogen to fluoroquinolone therapy.

Currently used fluoroquinolone antibiotics demonstrate limited effectiveness against infections caused by *Streptococcus pneumoniae*. The MIC₉₀ at which 90% of the isolates are inhibited (the MIC) of the most frequently prescribed fluoroquinolone, ciprofloxacin, for clinical isolates of *S. pneumoniae* is 1 to 3 µg/ml, which places this pathogen very close to the accepted breakpoint separating sensitive and resistant bacterial strains (4 µg/ml) (4). Here we demonstrate that the activity of a multidrug efflux transporter is likely to contribute to the intrinsically low sensitivity of *S. pneumoniae* to fluoroquinolones.

Several chromosomally encoded multidrug transporters have recently been identified in gram-positive bacteria: Bmr (8), Blt (2), and Bmr3 (10) of *Bacillus subtilis*; NorA (9, 17) of *Staphylococcus aureus*; LmrP (3) and LmrA (16) of *Lactococcus lactis*; and LfrA (14) of *Mycobacterium smegmatis*. These transporters recognize a wide variety of compounds, including fluoroquinolones and toxic hydrophobic cations, such as ethidium, and pump them out of cells (reviewed in reference 12). Many of these transporters can be inhibited by the plant alkaloid reserpine (1–3, 8, 9, 16), which, at least in one case, has been shown to interact directly with a multidrug transporter (1).

The idea that *S. pneumoniae* may also express a multidrug transporter came from an observation that the susceptibility of this pathogen to fluoroquinolones and ethidium significantly increases in the presence of reserpine. The rough laboratory strain CP1200 (7) of *S. pneumoniae* was inoculated at an optical density at 550 nm of 0.003 into wells of a 96-well microtiter plate containing 1:1.5 dilutions of drugs in CAT medium (13) or CAT medium containing reserpine at 10 µg/ml, and after 6 h of incubation at 37°C, bacterial growth was assessed by observing medium turbidity. Reserpine, by itself, did not affect the growth rate of these bacteria (not shown). However, as Fig. 1 demonstrates, the MICs of ethidium and of two fluoroquinolones, norfloxacin and ciprofloxacin, were 2.25 to 3.5 times lower in the presence of reserpine than in its absence,

suggesting the existence of a reserpine-sensitive drug defense mechanism(s) in *S. pneumoniae*.

In order to select cells overexpressing this hypothetical defense mechanism, we grew the CP1200 cells for 2 weeks in a series of gradually increasing concentrations of ethidium bromide (0.8 to 3.5 µg/ml) in CAT medium, after which they were plated and one of the clones, designated EBR, was analyzed further. The MIC of ethidium for the EBR strain was found to be 7.5 times higher than for the parental strain, CP1200, whereas reserpine completely reversed this resistance (Fig. 1).

In order to analyze the biochemical nature of the ethidium resistance, we monitored the kinetics of ethidium accumulation and efflux in the CP1200 and EBR cells by the fluorimetric method (3, 6, 8, 9, 16). As Fig. 2A demonstrates, the EBR cells accumulated ethidium to a much lower level than the CP1200 cells. Upon addition of reserpine, however, the rates of ethidium accumulation increased for both types of cells and became identical. With reserpine present, both types of cells accumulated equal amounts of ethidium (not shown). This result demonstrates that the reserpine-sensitive resistance to ethidium in the EBR cells is likely due to reduced accumulation of this toxic dye.

The reason for the reduced accumulation is the enhanced active efflux of ethidium in the EBR strain. When cells pre-

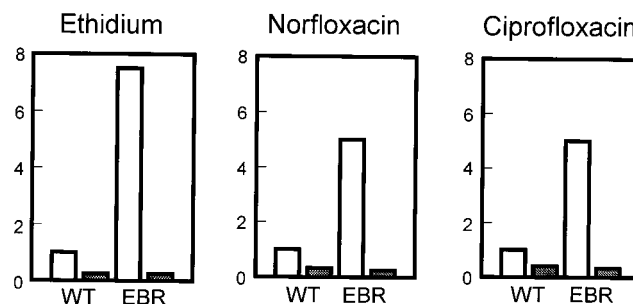


FIG. 1. Relative MICs of different drugs for the CP1200 (WT) and the ethidium-selected EBR strains of *S. pneumoniae* in the absence (open bars) and presence (filled bars) of 20 µM reserpine. The MIC for the CP1200 strain in the absence of reserpine is assumed to be 1. The actual MICs for this strain were as follows: ethidium, 0.9 µg/ml; norfloxacin, 2.2 µg/ml; and ciprofloxacin, 0.45 µg/ml.

* Corresponding author. Mailing address: M/C 870, University of Illinois, 900 S. Ashland Ave., Chicago, IL 60607. Phone: (312) 996-7231. Fax: (312) 413-9303. E-mail: neyfakh@uic.edu.

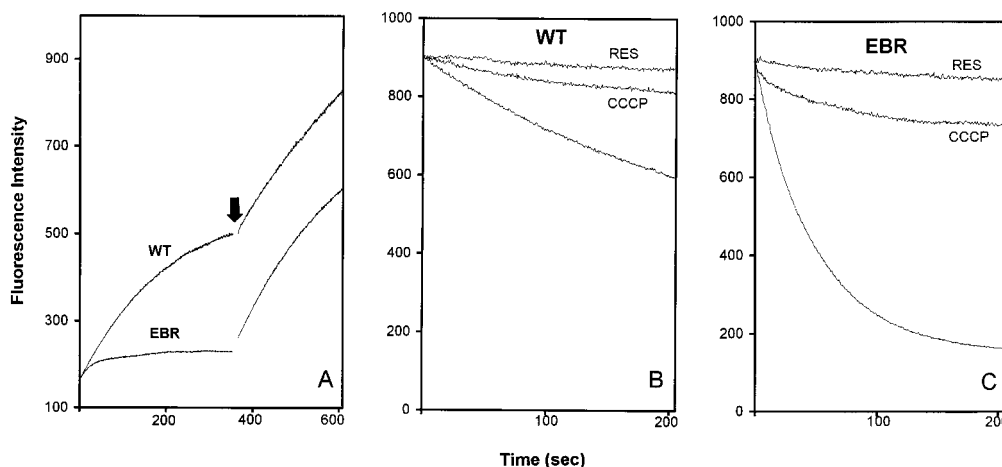


FIG. 2. Kinetics of accumulation (A) and efflux (B and C) of ethidium bromide by the CP1200 (WT) and the EBR strains of *S. pneumoniae*. Fluorescence intensity (in arbitrary units) is a measure of the amount of ethidium inside the cells (the fluorescence of this dye increases dramatically upon binding to intracellular components). (A) Cells grown in CAT medium were pelleted and resuspended to an optical density at 550 nm of 0.2 in a buffer containing 0.1 M morpholinepropanesulfonic acid (MOPS) (pH 7.0) and 0.2% glucose. Fluorescence recording (Shimadzu fluorimeter RF-1501 [λ_{ex} , 530 nm; λ_{em} , 600 nm]) starts with the addition of ethidium bromide to a concentration of 2 $\mu\text{g/ml}$. The arrow indicates the addition of reserpine (10 $\mu\text{g/ml}$). (B and C) Cells were preloaded for 20 min with ethidium bromide (2 $\mu\text{g/ml}$) in the presence of reserpine (RES) (10 $\mu\text{g/ml}$), quickly washed by centrifugation, and resuspended in the same buffer containing either no drugs, reserpine (10 $\mu\text{g/ml}$), or 50 μM CCCP, a membrane protonophore. Ethidium-selected EBR cells accumulate less ethidium and release it faster than the parental CP1200 cells. Reserpine inhibits ethidium efflux and stimulates its accumulation in both strains. Ethidium efflux is also inhibited by CCCP, suggesting that it is an energy-dependent process.

loaded with ethidium in the presence of reserpine were placed into fresh medium, they released the dye, with the EBR cells doing this much faster than the CP1200 cells (compare Fig. 2B and C). Importantly, this release was energy dependent: the membrane protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) dramatically reduced the rates of ethidium release. Furthermore, the efflux of ethidium from cells of both strains could be almost completely inhibited by reserpine. Taken together, the kinetics results indicate that *S. pneumoniae* expresses a reserpine-sensitive, energy-dependent transporter which promotes efflux of ethidium from cells, with the activity of this transporter being enhanced in the ethidium-selected EBR strain.

Importantly, as Fig. 1 demonstrates, the EBR strain acquired resistance not only to the selective agent, ethidium, but also to norfloxacin and ciprofloxacin, compounds unrelated to ethidium either by their chemical structure or by the mechanism of antibacterial action: their MICs for the EBR strain were five times higher than for the parental CP1200 strain. As was the case with ethidium, fluoroquinolone resistance could be completely reversed by reserpine (Fig. 1). No cross-resistances to ampicillin, chloramphenicol, erythromycin, novobiocin, or rifampin were observed in the EBR cells (not shown).

Although formally these results can be explained by assuming that the EBR cells express two separate mechanisms of drug defense, a reserpine-sensitive ethidium efflux transporter and an independent, yet also reserpine-sensitive, mechanism of fluoroquinolone resistance, it seems much more likely that these cells express a single reserpine-sensitive transporter, which, similarly to the already-known multidrug transporters of gram-positive bacteria, promotes efflux of both ethidium and fluoroquinolones.

It is clear from Fig. 1 that the activity of this multidrug transporter confers approximately 2.5-fold resistance to norfloxacin and ciprofloxacin upon wild-type *S. pneumoniae*. Overcoming even this relatively low level of intrinsic resistance would place the MIC₉₀s of fluoroquinolones for *S. pneumoniae* well below the boundary between susceptible and resistant clinical categories. Circumvention of the transporter activity

may also help in combating the acquired fluoroquinolone resistance developing in the drug-treated *S. pneumoniae* strains. Although this resistance has been attributed so far only to mutations in the targets of fluoroquinolone action, gyrase and topoisomerase IV (5, 11, 15), an active efflux mechanism may be an additional factor in the acquired resistance.

The gene encoding the *S. pneumoniae* multidrug transporter remains to be cloned and characterized. However, the EBR strain, which apparently overexpresses this transporter, can already be used either for identifying fluoroquinolones which are not susceptible to the transporter-mediated efflux or for developing clinically useful transporter inhibitors which, like reserpine, would potentiate fluoroquinolone action against *S. pneumoniae*.

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